Enteric glia modulate epithelial cell proliferation and differentiation through 15-deoxy-$\Delta^{12,14}$-prostaglandin J2

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The enteric nervous system (ENS) and its major component, enteric glial cells (EGCs), have recently been identified as a major regulator of intestinal epithelial barrier functions. Indeed, EGCs inhibit intestinal epithelial cell (IEC) proliferation and increase barrier resistance and IEC adhesion via the release of EGC-derived soluble factors. Interestingly, EGC regulation of intestinal epithelial barrier functions is reminiscent of previously reported peroxisome proliferator-activated receptor γ (PPARγ)-dependent functional effects. In this context, the present study aimed at identifying whether EGC could synthesize and release the main PPARγ ligand, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2), and regulate IEC functions such as proliferation and differentiation via a PPARγ dependent pathway. First, we demonstrated that the lipocalin but not the haematopoetic form for prostaglandin D synthase (PGDS), the enzyme responsible of 15dPGJ2 synthesis, was expressed in EGCs of the human submucosal plexus and of the subepithelium, as well as in rat primary culture of ENS and EGC lines. Next, 15dPGJ2 was identified in EGC supernatants of various EGC lines. 15dPGJ2 reproduced EGC inhibitory effects upon IEC proliferation, and inhibition of lipocalin PGDS expression by shRNA abrogated these effects. Furthermore, EGCs induced nuclear translocation of PPARγ in IEC, and both EGC and 15dPGJ2 effects upon IEC proliferation were prevented by the PPARγ antagonist GW9662. Finally, EGC induced differentiation-related gene expression in IEC through a PPARγ-dependent pathway. Our results identified 15dPGJ2 as a novel glial-derived mediator involved in the control of IEC proliferation/differentiation through activation of PPARγ. They also suggest that alterations of glial PGDS expression may modify intestinal epithelial barrier functions and be involved in the development of pathologies such as cancer or inflammatory bowel diseases.

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Abbreviations 15dPGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; EGC, enteric glial cell; EGC CM, enteric glial cell conditioned medium; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; GSNO, *S*-nitrosoglutathione; H-PGDS, haematopoietic-PGDS; L-PGDS, lipocalin-prostaglandin D synthase; PGDS, prostaglandin D synthase; PPARγ, peroxisome proliferator-activated receptor γ; TGFβ1, transforming growth factor β1.

Introduction

The intestinal epithelial barrier is a highly dynamic and specialized system. It is continuously renewed by processes involving cell proliferation, differentiation and migration from the stem cell compartment, and the extrusion of terminally differentiated cells into the intestinal lumen. As these cells emerge from the crypts and migrate along the crypt–villus axis, they cease to proliferate and acquire differentiated functions (Sancho *et al.* 2003; Nakamura *et al.* 2007). This balance between proliferation and

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differentiation is controlled by key signalling pathways within intestinal epithelial cells (IECs). It is also modulated by direct or indirect interactions between IECs themselves or with cellular and/or molecular constituents of their microenvironment. Besides canonical pathways such as Wnt/βcat or Notch, recent data have shown that peroxisome proliferator-activated receptor γ (PPARγ) play a key role in controlling IEC functions (Nakamura *et al.* 2007; Su *et al.* 2007). PPARγ is a nuclear receptor highly expressed in the gut and in particular in the colon (Lefebvre *et al.* 1999). For instance, PPARγ activation inhibits IEC proliferation and promotes cell differentiation (Sarraf *et al.* 1998; Kitamura *et al.* 1999; Wachtershauser *et al.* 2000; Kato *et al.* 2004).

Although the cellular sources of PPARγ natural ligands need currently to be better defined, PPARγ ligands, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2), can be provided by nutritional factors or can be synthesized by endogenous sources (Bull *et al.* 2003). In particular, 15dPGJ2 is derived from prostaglandin D2, which is produced from prostaglandin H2 by prostaglandin D synthase (PGDS).

Among the constituents of the intestinal epithelial barrier microenvironment, the enteric nervous system (ENS), and in particular enteric glial cells (EGCs), is involved in the regulation of intestinal epithelial barrier functions (Cabarrocas *et al.* 2003; Neunlist *et al.* 2008). EGCs form a dense network of cells in close proximity to IECs, allowing paracrine interactions with IECs. Recent anatomical and functional evidence has pointed out their major role in intestinal epithelial barrier control (Savidge *et al.* 2007). In particular, EGCs increase barrier resistance (Neunlist *et al.* 2007), inhibit IEC proliferation (Neunlist *et al.* 2007) and increase IEC adhesion (Van Landeghem *et al.* 2009). These studies have identified various glial mediators involved such as TGFβ1 and *S*-nitrosoglutathione (GSNO) (Neunlist *et al.* 2007; Savidge *et al.* 2007). Although some of the EGC effects on IECs are reminiscent of PPARγ activation, it remains currently unknown whether EGCs can synthesize PPARγ ligands such as 15dPGJ2. Whether these effects on IEC functions are mediated through PPARγ also remains to be elucidated.

In this context, our study aimed at demonstrating that EGCs could be a source of 15dPGJ2 and that EGCs regulate IEC proliferation and differentiation through PPARγ-dependent pathway.

Methods

Human tissues

Tissue specimens were obtained from patients who underwent surgery for colonic adenocarcinoma or in human mucosal biopsies of patients who had colonoscopy. For biopsy studies, patients gave their informed consent. Specimens were taken at a distance from the tumour in macroscopically and histologically normal areas and immediately processed in the Pathology Department. According to the guidelines of the French Ethics Committee for Research on Human Tissues, these specimens were considered as 'residual tissues', not relevant to pathological diagnosis. Tissue samples were subsequently fixed in 4% paraformaldehyde for 3 h at room temperature. Following several washes in phosphate-buffered saline (PBS), the tissue was pinned and whole mounts of mucosal and submucosal plexus were obtained by microdissection under a SZ3060 microscope (Olympus, Rungis, France), as previously described (Neunlist *et al.* 2007).

Primary culture of enteric nervous system

Primary culture of rat ENS was performed as previously described (Chevalier *et al.* 2008). Experiments were complied with Journal policies and UK regulations on animal experimentation as described by Drummond (2009). Pregnant Sprague–Dawley rats were purchased (CERJ, Le Genest St Isle, France) and manipulated in compliance with the French institutional guidelines. These procedures were approved by the local institutional animal research committee (Agreement E. 44011; Inserm, Nantes, France). Every effort was made to minimize animal suffering and the number of animal used. Rats were killed by an overdose of CO_2 followed by severing the carotid arteries. Intestinal cells obtained after dissection and dissociation were counted and then seeded at a density of 2.4×10^5 cells cm^{-2} on 24-well plates (Corning, Avon, France) previously coated with a 0.5% gelatin solution (Sigma-Aldrich, Lyon, France). Primary culture was maintained for 14 days with half of the medium (Dubelcco's modified Eagle's medium–F_{12} 1:1 containing 1% of N-2 supplement (Life Technologies, Cergy Pontoise, France)) replaced every day.

Enteric glial cells

Non-transformed or transformed EGCs were obtained as previously described (Ruhl *et al.* 2001). JUG2 was obtained from ENS primary culture derived from rat embryonic intestine (Chevalier *et al.* 2008). After 13 days of culture, primary cultures were trypsinized and seeded in serum-containing medium after differential centrifugation. Following 7 days of culture, isolated areas of cells that resembled morphological glial cells were trypsinized using a cloning cylinder and seeded in a culture flask in serum-containing medium. After 1 month, the cells were immunoreactive for glial but not for neuronal or myofibroblast markers. ECGs

were cultured in Dubelcco's modified Eagle's medium (4.5 g l^{-1} glucose; Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Biowest, Nuaillé, France), 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Life Technologies). EGCs were seeded at a density of 50,000 cells cm^{-2} in 12-well plates (Corning). Cells were grown to confluence (for 3 days). To assess the role of EGC conditioned medium (EGC CM), upon IEC proliferation, EGCs were first cultured for 24 h or 48 h after they reached confluency. Then, EGC culture medium was collected, centrifuged at 1000 *g* for 10 min and used immediately. For immunohistochimistry, rat EGCs and primary cultures of rat ENS were first fixed in 4% paraformaldehyde. Then, immunofluorescence staining was performed as described below.

Lentiviral vectors production and transduction of EGCs

The plasmid vectors TRC1-pLKO.1-puro were purchased from Sigma (Mission shRNA library). Plasmid clones expressing a shRNA targeting the lipocalin-prostaglandin D synthase (L-PGDS) mRNA (pLKO.1-puro-sh913) and a non-functional L-PGDS shRNA vector used as a negative control (pLKO.1-puro-sh917), were used to transfect 293T cells. Two other plasmid clones were kindly provided by Dr Patrick Salmon (Department of Neuroscience, Faculty of Medicine, University of Geneva, Geneva, Switzerland). The psPax2 packaging plasmid vector expresses the complete sets of structural and accessory viral proteins with the exception of env and vpu, and is devoid of the packaging sequence ψ The plasmid pMD2-G expresses the vesicular stomatitis virus G envelope protein allowing efficient pseudotyping of lentiviral particles and broad cell tropism. Lentiviral vectors particles were generated upon cotransfection of 293T cells with 30 μg transfer vector (TRC1-pLKO.1-puro), and 22.5 μg psPax2 and 15 μg pMD2G, using the transient calcium phosphate method as described previously (Naldini *et al.* 1996). Briefly, 6×10^6 293T cells per 150 cm^2 tissue culture dish (Costar, Brumath, France) were seeded 24 h prior to transfection. Viral supernatants were harvested 48 h post-transfection, cleared by low-speed centrifugation and filtrated using a 45 μm filter (Millipore, Saint-Quentin en Yvelines, France). The vector was concentrated by centrifugation at 47,900 *g* for 3 h at 4°C and the viral pellet resuspended in 250 μl of cold advanced alpha-Dubelcco's modified Eagle's medium and stored at −80°C until use.

Cell transductions were carried out on 5000 EGCs cultured with 200 μl fetal calf serum free culture medium and seeded 24 h prior to transduction. The lentivirus was diluted at a concentration of 1/100 (from the prepared stock solution). After overnight incubation at 37°C, culture medium was replaced by fresh EGC medium complemented with 10% fetal calf serum. EGC lines were then grown in medium supplemented with 2 μg ml^{-1} puromycin (Sigma). Upon selection, resistant EGCs were expanded and tested for L-PGDS expression to select the best clones.

Coculture model and treatment of intestinal epithelial cells with EGC CM

Caco-2 epithelial cells were seeded on Transwell filters at a density of 70,000 cells cm^{-2} and EGCs were seeded in the wells of 12-well plates. For coculture experiments, IECs on Transwell filters were placed on the top of wells containing EGCs. In other set of experiments, culture medium with or without 15dPGJ2 5 μM (Sigma), EGC CM with or without the potent and irreversible PPARγ inhibitor GW9662 10 μM (Sigma) (Gupta *et al.* 2001) were added to IECs cultured on Transwell filters. Additional experiments were performed with other IEC lines, i.e. T84 and IEC6, cultured as previously described (Neunlist *et al.* 2007). For transcriptional studies, these experimental conditions were maintained for 24 h. For cell growth studies, these conditions were maintained for 6 days. Half of the culture medium was changed daily.

15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 extraction and measurement

Measurement of 15dPGJ2 in EGC CM was performed as previously described (Thevenon *et al.* 2001). Appropriate amounts of internal standard 15dPGJ2-d4 were added to samples before prostaglandin extraction with ethyl acetate. Resulting extracts were dried and treated with 2,3,4,5,6-pentafluorobenzyl bromide to convert 15dPGJ2 into its derivative. Derivative was then quantified by gas chromatography associated with mass spectrometry with the negative ion chemical ionization mode on the single ion monitoring basis (m/Z = M-181). This was carried out with an Agilent Technologies gas chromatograph (model 6890) interfaced with a quadrupole mass spectrometer (model 5973). The chromatograph was equipped with an HP-1MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 mm film sickness) at 57°C. Helium and methane were used as carrier and reagent gases, respectively.

Cell counting

For cell growth studies, IECs were harvested with 1% trypsin-EDTA (Life Technologies) and homogenized. Cells were counted in a blinded fashion using Malassez slides (VWR International, Strasbourg, France) and fluorescence activated cell sorting (BD FACS, BD Bioscience, Erembodegem, Belgium). Cell death was estimated using trypan blue assay and

7-aminoactinomycin D (BD Biosciences) incorporation using FACS analysis.

Immunofluorescence staining

For immunofluorescence staining, all antibodies were diluted in PBS with 1 mg ml^{-1} sodium azide, 4% horse serum and 1% Triton X-100. Whole mounts of human submucosa and mucosa were incubated for 24 h with PBS sodium azide horse serum and Triton X-100 followed by incubation with rabbit polyclonal anti-PGDS (1:500; Cayman, Spi-Bio, Montigny-le-Bretonneux, France; raised against human PGDS) overnight at 4°C. After extensive rinsing in PBS, whole mounts were incubated with donkey anti-rabbit IgG conjugated with carboxy-methyl-indocyanine (1:500; Beckman Coulter, Roissy, France) or Alexa Fluor 488 (1:400; Molecular Probes, Invitrogen, Cergy-Pontoise, France) for 3 h at room temperature. To show the specificity of the PGDS staining, the antibody was first pre-incubated with the PGDS blocking peptide (w/w, Cayman) for 1 h at room temperature. Next, EGC were incubated with the antibody-blocking peptide solution overnight at 4°C and then stained with the secondary antibody as described below. After PGDS staining, the whole mount was incubated with goat polyclonal anti-S100β (1:100; Santa-Cruz, Tebu-bio, Le Perray en Yveline, France) overnight at 4°C followed by donkey anti-goat IgG conjugated with Alexa Fluor 488 (1:400; Molecular Probes) or carboxy-methyl-indocyanine (1:500; Beckman Coulter) for 3h at room temperature. After PGDS staining, primary culture was incubated with mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:100) overnight at 4°C followed by donkey anti-mouse IgG conjugated with Alexa Fluor 488 fluorescent dye (1:500; Molecular Probes). Following washes, stained samples were observed and acquired with a microscope IX 50 (Olympus) or with the Axiovert 200M microscope coupled to Apotome (Zeiss, Göttingen, Germany).

Real-time RT-PCR analysis

Total RNA was extracted from Caco-2 cells, EGC cells or human mucosal biopsies using the Qiamp total RNA kit (Qiagen, Courtaboeuf, France) and cDNA was synthetized using standard procedures as previously described (Neunlist *et al.* 2007) and real-time PCR was performed. Primers were designed from the sequence of the human or the rat cDNAs using the Universal ProbeLibrary Assay design Center (https://roche-applied-science.com/sis/rtpcr/url). The sequences of these primers are listed in Table 1. PCR amplifications were performed using the LC480 SYBR Green I master mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol and run on a Rotogene 3000 instrument (Corbett Research, Courtaboeuf, France). Expression of the S6 and GAPDH genes was analysed in parallel as an internal control. Each RT-qPCR reaction for L-PGDS mRNA was directly loaded onto non-denaturing 2% agarose gels, stained with SYBR safe (Invitrogen), and visualized under UV trans-illumination. Specificity of the primers was determined by sequencing the amplification products.

Western blot

EGCs were harvested in RIPA buffer (Millipore) containing 2 mM sodium orthovanadate and proteases inhibitors (Roche, Meylan, France). Equal amounts of lysate were separated using NuPage Novex Bis Tris MiniGels (Invitrogen) before electrophoretic transfer using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (100 mM NaCl, 10 mM Tris, pH 7.5) containing 5% non-fat dry milk. Membranes were incubated overnight at 4°C with rabbit anti-L-PGDS primary antibodies (1:1000, Cayman). Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Amersham, Saclay, France; diluted 1:5000) and visualized by enhanced chemiluminescence detection (ECLplus, Amersham).

PPARγ nuclear translocation assays

To examine PPARγ nuclear translocation in IECs, indirect immunofluorescence staining was performed, using antibodies directed against human PPARγ (Calbiochem, Darmstadt, Germany). Following coculture or treatment of IECs, filters were fixed in 4% paraformaldehyde for 30 min. Immunofluorescence staining was then performed as described above using rabbit polyclonal anti-PPARγ antibody (1:1000) for 1 h. After extensive washing with PBS, filters were incubated with goat anti-rabbit antibodies conjugated with carboxy-methyl-indocyanine (1:500; Beckman Coulter) and 4′,6′-diamidino-2-phenylindole (1 mg l^{-1} in phosphate buffer; Merck, Fontenay-sous-Bois, France) for 30 min. Filters were examined with an IX50 microscope (Olympus) as described above.

Statistical analysis

Statistical analyses were performed using Prism 4.0 (GraphPad Software Inc., La Jolla, USA). Experimental data were compared using a non-parametric Mann–Whitney test or Kruskall–Wallis test followed by Dunn's *post hoc* test. A P value < 0.05 was considered to be significant.

Table 1. Primers for real time RT-PCR analysis

Primer sequences (5′-3′)	Size of the amplicon (bp)	Annealing temperature (°C)	Acquisition temperature (°C)
hPPARγ (NM_138712)			
Fw: 5′-TTGCTGTCATTATTCTCAGTGGA-3′	124	63	82
Rev: 5′-GAGGACTCAGGGTGGTTCAG-3′			
hALPI (NM_001631)			
Fw: 5′-TTTGGTGGCTACACCTTGC-3′	92	63	85
Rev: 5′-CCGTACAGGATGGACGTGT-3′			
hGAPDH (NM_002046)			
Fw: 5′-CTGACTTCAACAGCGACACC-3′	109	63	80
Rev: 5′-TAGCCAAATTCGTTGTCATACC-3′			
hRPS6 (NM_001010)			
Fw: 5′-CCAAGCTTATTCAGCGTCTTGTTACTCC-3′	146	60	86
Rev: 5′-CCCTCGAGTCCTTCATTCTCTTGGC-3′			
rL-PGDS (NM_013015)			
Fw: 5′-GGATTTCCACAGACCCCAGC-3′	130	60	84
Rev: 5′-GCTCTTTCTTCTCCCGGAACC-3′			
hL-PGDS (NM_000954)			
Fw: 5′-AGAAGAAGGCGGCGTTGTCC-3′	195	60	84
Rev: 5′-CCACCACTGACACGGAGTAGG-3′			
rH-PGDS (NM_031644)			
Fw: 5′-CACAGAATAGAACAAGCTGACTGG-3′	133	62	84
Rev: 5′-CTGTGTTTTTGGTCAAATATCTTGC-3′			
hH-PGDS (NM_014485)			
Fw: 5′-CACAGAATAGAACAAGCTGACTGG-3′	133	62	84
Rev: 5′-CTGTGTTTTTGGTCAAATATCTTGC-3′			
rRPS6 (NM_017160)			
Fw: 5′-CCAAGCTTATTCAGCGTCTTGTTACTCC-3′	146	60	86
Rev: 5′-CCCTCGAGTCCTTCATTCTCTTGGC-3′			

Results

EGCs express L-prostaglandin D synthase

We first examined whether EGCs express PGDS, the main enzyme responsible for 15dPGJ2 synthesis. By immunostaining, we found that PGDS was expressed in rat EGC lines (Fig. 1*A*) and in EGCs of rat ENS primary culture (Fig. 1*B*). The specificity of the PGDS staining was validated using a PGDS blocking peptide (Fig. 1*A*). Furthermore, PGDS was expressed in EGCs of colonic human submucosal plexus (Fig. 1*C*) as well as in some subepithelial EGCs closely surrounding colonic crypts (Fig. 1*D*).

As two forms of PDGS have been reported, the lipocalin form of PGDS (L-PGDS) and the haematopoietic form (H-PGDS), we performed transcriptomic studies to characterize their respective expression in EGCs. Using RT-PCR, we found that L-PGDS was expressed in rat EGC lines (Fig. 2*A*), in primary culture of rat ENS (Fig. 2*B*) and in colonic biopsies of healthy subjects (Fig. 2*C*). In contrast, H-PGDS was not expressed in EGC lines but was present in Caco-2 (data not shown). Interestingly, we found a highly and significantly positive correlation between L-PGDS mRNA and S100β mRNA expression in human colonic biopsies ($n = 11$, $r = 0.90$; $P = 0.01$; Fig. 2*D*). No mRNA expression of the L-PGDS form was detected in the other cell lines used in this study (Caco-2 and CCD 18Co; data not shown).

EGCs are a source of 15dPGJ2, natural PPARγ agonist

To determine if EGCs can produce PPARγ natural agonists, EGC CM was analysed using gas chromatography associated with mass spectrometry. We were able to identify 15dPGJ2 in the supernatant of transformed (Fig. 3) and non-transformed EGCs (data not shown).

EGCs inhibit IEC proliferation via the release of 15dPGJ2

After 6 days, EGCs or EGC CM significantly inhibited Caco-2 cell number by 60% and 40% as compared to control, respectively (Fig. 4). To determine whether inhibition of IEC proliferation by EGCs was mediated by glial secretion of 15dPGJ2, we inhibited L-PGDS in EGC by shRNA. L-PGDS mRNA (Fig. 5*A*) and protein (Fig. 5*B*) expression were significantly decreased in EGC transduced with L-PGDS antisense shRNA (EGC

shPGDS) as compared to cells treated with a non functional-shRNA (EGC Mock). The antiproliferative effects of EGCs were abolished in EGC shPGDS as compared to EGC or EGC Mock (Fig. 5*C*). Finally, we showed that 15dPGJ2 (5 μM) reduced Caco-2 cell number by 30% (Fig. 6).

EGC and 15dPGJ2 effect in IEC involve PPARγ-dependent pathways

In order to determine whether EGCs can activate PPARγ in Caco-2 cells, we studied PPARγ nuclear translocation in these cells following coculture with EGCs. While PPARγ localisation was cytoplasmic in control condition (Fig. 6*A*), a nuclear localisation was observed after a 24 h coculture both with EGCs (Fig. 6*B*) and EGC CM (Fig. 6*C*). This effect was similar to that obtained when Caco-2 cells were cultured in the presence of rosiglitazone, a synthetic PPARγ agonist (data not shown). Finally, GW9662 (10 μM), a PPARγ antagonist, significantly inhibited the anti-proliferative effects of both EGC CM and 15dPGJ2 on IECs (Fig. 6*D*).

In addition, the anti-proliferative effects of EGCs and EGC CM were observed in T84 and IEC6 (changes in IEC number for T84 and IEC6 as compared to control: EGC 61%; EGC CM 78% ($n = 2$) and EGC 36%; EGC CM 59% ($n = 3$), respectively). In both IEC lines, GW9662 (10 μM) also inhibited the anti-proliferative effect of EGC CM (changes in IEC number for T84 and IEC6 as compared to control: EGC CM 78%; EGC CM+GW9662 90% ($n = 2$) and EGC CM 59%; EGC CM+GW9662 83% ($n = 3$), respectively).

EGCs induce differentiation-related genes expression in IECs through the PPARγ pathway

To determine whether EGC-induced changes in cell number were associated with IEC differentiation, we examined the effects of EGCs on mRNA expression in Caco-2 cells of differentiation-related genes such as PPARγ, E-cadherin and intestinal alkaline phosphatase (hALPI).

EGC CM significantly upregulated PPARγ, E-cadherin and hALPI mRNA expression in Caco-2 cells after 24h

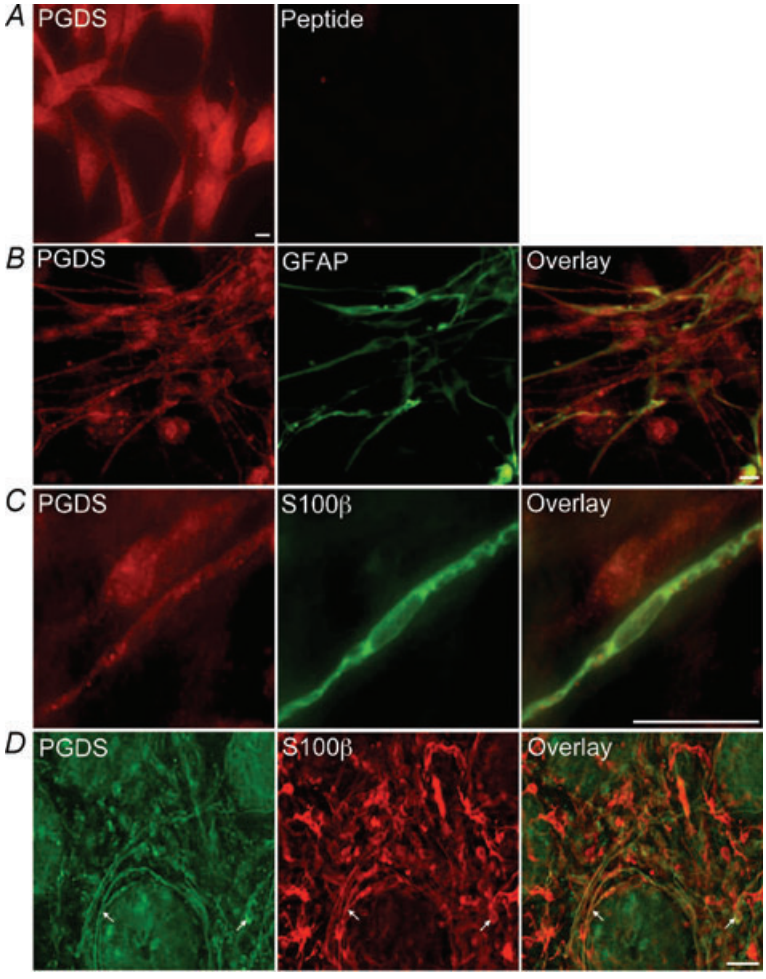

Figure 1. Enteric glial cells express prostaglandin D synthase
Immunofluorescence staining of prostaglandin D synthase (PGDS) in rat enteric glial cell lines without and with blocking peptides (*A*), in primary culture of rat enteric nervous system (ENS) (*B*), in human submucosal plexus (*C*) and in human subepithelial layer (*D*). In primary culture of rat ENS (*B*), glial fibrillary acidic protein (GFAP) immunoreactive cells were colocalized with PGDS. In human submucosal plexus (*C*) and subepithelial layer (*D*), some S100β-immunoreactive cells were colocalized with PGDS (white arrows). Each picture is representative of five experiments. Scales bars are 5 μm (*A*–*C*) and 100 μm (*D*).

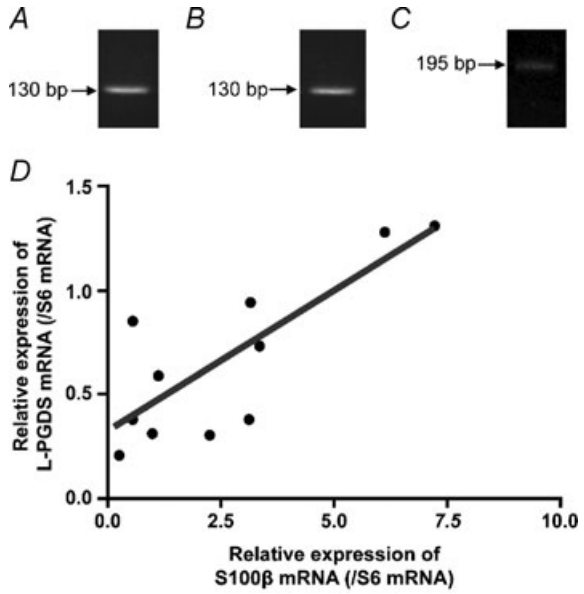

Figure 2. Transcriptional expression of lipocalin-prostaglandin D synthase in enteric glial cells
RT-PCR analysis of lipocalin-prostaglandin D synthase (L-PGDS) mRNA expression showed a 130 bp band in rat enteric glial cell lines (*A*) and in rat primary culture of enteric nervous system (*B*). In human colonic biopsies, a 195 bp band was detected (*C*). Quantitative PCR analysis of human colonic biopsies revealed a positive correlation between L-PGDS and S100β mRNA expression (*D*) ($n = 11$; $r^2 = 0.63$; $P = 0.01$; linear regression).

of culture. Interestingly, GW9662 treatment completely abrogated these effects (Fig. 7).

Discussion

A major finding of this study was the identification of EGC as a cellular source of the natural PPARγ ligand, 15dPGJ2. We further demonstrated that 15dPGJ2 mediated in part the anti-proliferative effects of EGC via PPARγ activation in IECs.

15dPGJ2 produced by EGCs was synthesized by L-PGDS but not H-PGDS. These two enzymes are encoded by two different genes (Urade & Eguchi, 2002). H-PGDS is a glutathione-requiring enzyme also named 'spleen' type (Christ-Hazelhof & Nugteren, 1979; Urade *et al.* 1987) and has been described in spleen, thymus, bone marrow, intestine, skin and stomach (Ujihara *et al.* 1988; Urade *et al.* 1989, 1990). L-PGDS is a glutathione-independent enzyme, also named 'brain' type, identified in the brain (Urade *et al.* 1985). The expression of L-PGDS but not H-PGDS in EGCs is reminiscent of what has been observed in glial cells of the central nervous system. Indeed, L-PGDS but not H-PGDS has been reported in the central nervous system in oligodendrocytes (Beuckmann *et al.* 2000) and in astrocytes in culture (Giacomelli *et al.* 1996). In contrast, in dorsal root ganglia, satellite cells and Schwann cells only

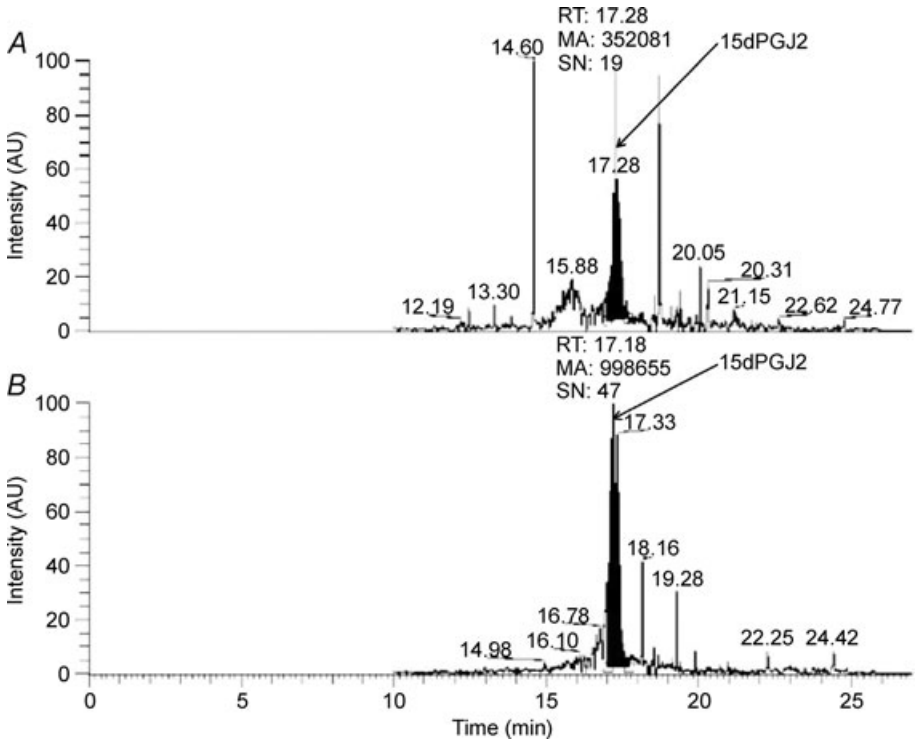

Figure 3. Enteric glial cells secrete 15-deoxy-$\Delta^{12,14}$-prostaglandin J2
Gas chromatography associated with mass spectrometry analysis showed the presence of 15dPGJ2 in transformed enteric glial cell conditioned medium (representative pattern of three experiments) (*A*). A control pattern was generated with Dubelcco's modified Eagle medium supplemented with 15dPGJ2 (*B*).

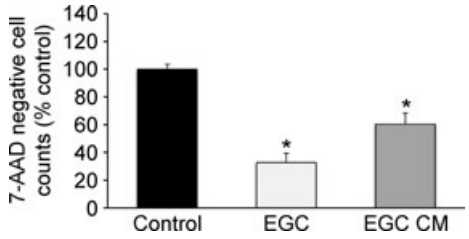

Figure 4. Enteric glial cells inhibit Caco-2 cell proliferation
Fluorescence activated cell sorting analysis of 7-aminoactinomycin D (7-AAD) negative Caco-2 cells showed that enteric glial cells (EGCs) or EGC conditioned medium (EGC CM) induced a significant decrease in Caco-2 cell number after 6 days of culture as compared to untreated (Control) Caco-2 monolayer.

express H-PGDS while L-PGDS expression is restricted to a subclass of neurons (Vesin *et al.* 1995*a*,*b*). These differences in PGDS type expression in dorsal root ganglia and in EGCs could be due to their different developmental origin or different glial cell subtypes (Dulac & Le Douarin, 1991). Indeed, our data also suggest that all EGCs do not express L-PGDS. Interestingly, in the colon besides EGCs, other cell types of the submucosa probably also express L-PGDS, as suggested in Fig. 1*C*. In particular, we observed L-PGDS expression in enteric neurons (data not shown), but its functional role remains to be identified.

Besides EGCs, other cell types of the gut produce 15dPGJ2, in particular by H-PGDS. Indeed, H-PGDS is the most representative form of PGDS in the gut and is expressed in IECs, mast cells and fibroblasts (Park *et al.* 2007; Hokari *et al.* 2009). This is consistent with our results showing the absence of L-PGDS mRNA expression in IECs and fibroblasts. However, expression of L-PGDS can be induced in these cells, for instance following infection with *H. pilory* (Hokari *et al.* 2009).

Since EGCs are major cellular constituents of the intestinal epithelial barrier microenvironment, we next determined the impact of EGC-derived 15dPGJ2 on IEC functions. EGCs densely innervate the intestinal epithelial barrier, surrounding intestinal crypts, and are located at a distance of less than 1 μm from the basolateral side of IECs (Mestres *et al.* 1992; Neunlist *et al.* 2007). Consistently, our study showing that subepithelial EGCs express L-PGDS set the anatomical basis for a paracrine action of EGC derived 15dPGJ2 on IECs. EGCs are known to inhibit cell proliferation and reduce paracellular permeability via

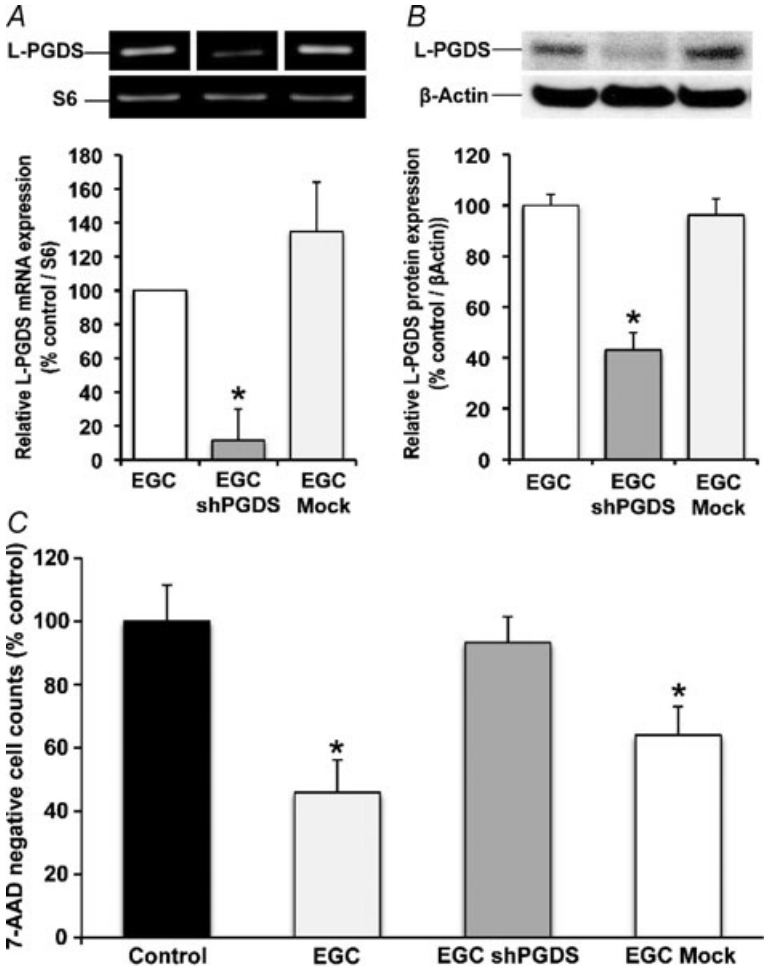

Figure 5. Transduction of EGC with L-prostaglandin D synthase shRNA abrogate their anti-proliferative effects
RT-qPCR analysis showed that lipocalin-prostaglandin D synthase (L-PGDS) mRNA expression was significantly decreased in L-PGDS shRNA transduced enteric glial cells (EGC shPGDS) as compared to mock-transduced EGCs (EGC Mock) and non-transduced EGCs (EGC) ($n = 5$, Kruskall–Wallis test followed by Dunn's *post hoc* test; $P < 0.05$) (*A*). Western blot analysis showed that L-PGDS protein expression was significantly decreased in L-PGDS shRNA transduced enteric glial cells (EGC shPGDS) as compared to mock-transduced EGCs (EGC Mock) and non-transduced EGCs (EGC) ($n = 7$, Kruskall–Wallis test followed by Dunn's *post hoc* test; $P < 0.05$) (*B*). Fluorescence activated cell sorting analysis of 7-aminoactinomycin D (7-AAD) negative Caco-2 cells showed that coculture of Caco-2 cells with EGCs or EGC Mock but not EGC shPGDS induced a significant decrease in Caco-2 cell number as compared to control. Values are expressed as means ± S.E.M. ($n = 4$; Kruskall–Wallis test followed by Dunn's *post hoc* test; $P < 0.05$) (*C*).

secretion of soluble factors such as TGFβ1 and GSNO, respectively (Neunlist *et al.* 2007; Savidge *et al.* 2007). These mediators were only partly responsible for the effects observed suggesting that other mediators were involved. In this context, we showed that EGC-derived 15dPGJ2 significantly reduced intestinal cell proliferation. In contrast, 15dPGJ2 had no effect upon paracellular permeability on differentiated Caco-2 monolayer (data

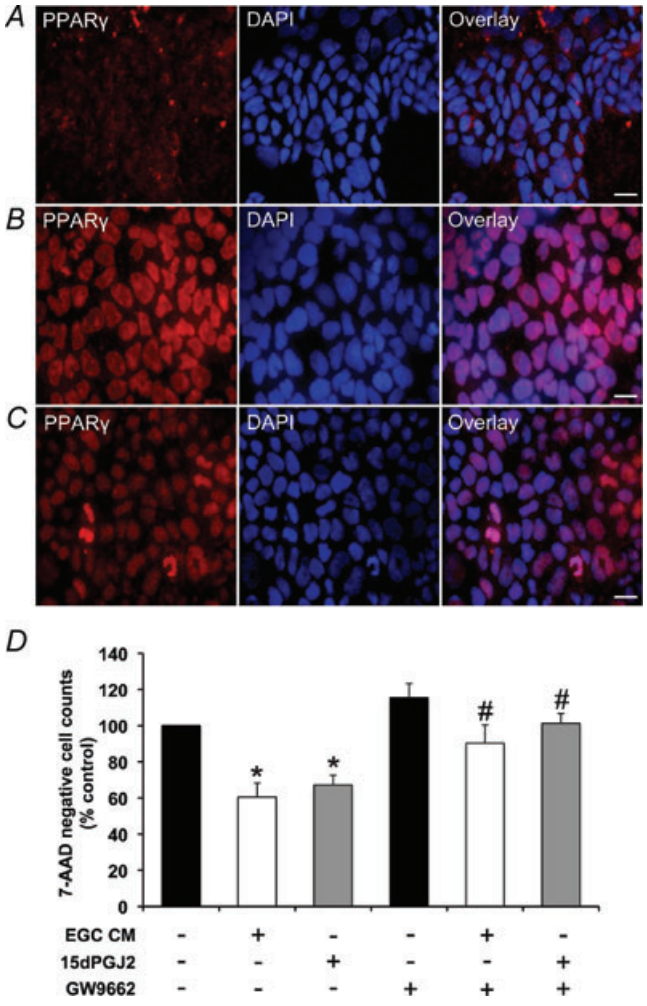

Figure 6. Effect of enteric glial cells on peroxisome proliferator-activated receptor γ translocation in Caco-2 cells
Immunofluorescence staining of peroxisome proliferator-activated receptor γ (PPARγ) in Caco-2 cells showed that it was uniformly distributed in the cytoplasm of cells cultured alone (*A*). In contrast, following coculture with enteric glial cells (*B*) or treatment with enteric glial cell conditioned medium (EGC CM) (*C*), PPARγ was mainly localized in the nucleus, identified by 4′,6′-diamidino-2-phenylindole (DAPI) staining. Data are representative of 3 independent experiments. Scale bars: 10 μm. *D*, fluorescence activated cell sorting analysis of 7-aminoactinomycin D (7-AAD) negative Caco-2 cells showed that EGC CM ($n = 6$) or 15dPGJ2 (5 μM; $n = 3$) induced a significant decrease in Caco-2 cell number as compared to control. PPARγ antagonist (GW9662, 10 μM) alone did not modify the cell number as compared to control. In the presence of GW9662, EGC CM ($n = 6$) or 15dPGJ2 (5 μM; $n = 3$) did not decrease Caco-2 cell number as compared to control. Values are expressed as means ± S.E.M. from 6 independents experiments (Kruskall–Wallis test followed by Dunn's *post hoc* test; $P < 0.05$ * as compared to non treated Caco-2; # as compared to Caco-2 treated with EGC CM or 15dPGJ2).

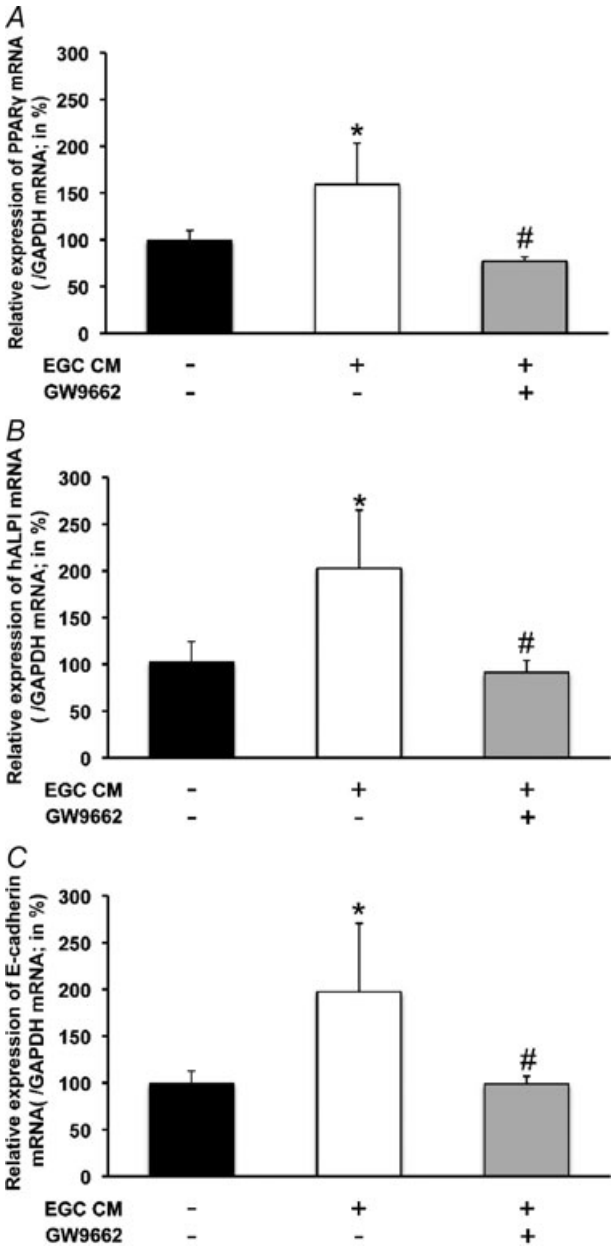

Figure 7. Enteric glial cells induce mRNA expression of differentiation marker genes
RT-qPCR analysis showed that mRNA expression of peroxisome proliferator-activated receptor γ (PPARγ) (*A*), intestinal alkaline phosphatase (hALPI) (*B*) and E-cadherin (*C*) was increased in Caco-2 cells cultured with enteric glial cell conditioned medium (EGC CM) as compared to Caco-2 cultured alone ($n = 7$). This effect was completely abrogated following culture with EGC CM in presence of GW9662 ($n = 3$). Values are expressed as means ± S.E.M. (Kruskall–Wallis test followed by Dunn's *post hoc* test; $P < 0.05$ * as compared to non treated Caco-2; # as compared to Caco-2 treated with EGC CM).

not shown), consistent with a recent report showing that under basal condition 15dPGJ2 does not modify paracellular permeability (Ponferrada *et al.* 2007).

Another important finding of this study is the demonstration that EGC effects upon IEC proliferation and differentiation are mediated in part through activation of PPARγ. EGCs induce a nuclear translocation of PPARγ in IECs and their functional effects are inhibited by a specific PPARγ antagonist. It is well known that, *in vitro*, treatment of IECs with PPARγ ligands induces cell-cycle blockade resulting in inhibition of cell proliferation and stimulation of cell differentiation (Kitamura *et al.* 1999; Gupta *et al.* 2001). Consistently, we have shown that EGCs induce a cell cycle blockade at G0/G1 in Caco-2 cells (Neunlist *et al.* 2008). In IECs, the cellular target of PPARγ, following activation by EGCs, remains unknown but could involve, for instance, Krüppel-like factor 4 (KLF4). Indeed, PPARγ activation modulates cell cycle regulators such as cyclin D1 or p21WAF1/Cip1, through the regulation of KLF4 (Koeffler, 2003; Rageul *et al.* 2009). KLF4 also plays major roles in IEC differentiation and maturation (Dang *et al.* 2000; Chen *et al.* 2003). In addition, EGC CM induced upregulation of mRNA expression of differentiation-related genes in IECs, i.e. hALPI (Hinnebusch *et al.* 2004), E-cadherin (Laprise *et al.* 2002) and PPARγ (Kato *et al.* 2004). These results are consistent with a recent transcriptomic study showing that EGCs up-regulated similar differentiation-related genes (Van Landeghem *et al.* 2009). Altogether these results suggest that EGCs promote IEC differentiation. Furthermore, these effects of EGCs were probably mediated by activation of PPARγ, as they were blocked by GW9662. The effects of EGCs on E-cadherin mRNA expression are in contrast with a previous study showing that EGCs did not modify E-cadherin protein expression (Savidge *et al.* 2007). This discrepancy could be explained in part by the experimental condition used. Savidge *et al.* (2007) characterized the impact of EGC in a nearly confluent IEC monolayer, probably constituted of differentiated cells. In contrast, in our study, IECs are in a proliferating state and could thus probably be more able to respond to differentiating signals induced by EGCs. Such a hypothesis is further supported by our results demonstrating that the modulation by EGCs of specific genes in IECs is dependent on their state of differentiation (data not shown).

In conclusion, these data add novel insights into the mechanisms by which EGCs are involved in the control of IEC functions. In particular, we demonstrate that EGCs secrete 15dPGJ2, which inhibits IEC proliferation and upregulates differentiation-related genes via activation of PPARγ. Furthermore, this study can be pursued to determine whether EGCs could also synthesize and release other natural PPARγ ligands which remain to be identified. This study provides new understanding for the role of EGCs in the modulation of IEC functions and has putative implications for early nutritional or pharmacological interventions in pathologies where the EGC network is altered, such as inflammatory bowel diseases or colorectal cancerous states.

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Author contributions

K.B.-N. and M.M.M. designed the study and carried out and experiments. P.A. and H.A. performed experiments. S.B. carried out lentiviral vectors production and performed EGC transduction. M.G.D., B.L. and A.B. contributed to the studies and gave their expertise in writing the paper. M.N., K.B.-N. and D.M. supervised the project and wrote the manuscript. All authors read and approved the final manuscript.

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